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Natural products against key *Mycobacterium tuberculosis* enzymatic targets: Emerging opportunities for drug discovery

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ABSTRACT

For centuries, natural products (NPs) have served as powerful therapeutics against a variety of human ailments. Nowadays, they still represent invaluable resources for the treatment of many diseases, including bacterial infections. After nearly three decades since the World Health Organization's (WHO) declaration of tuberculosis (TB) as a global health emergency, *Mycobacterium tuberculosis* (*Mtb*) continues to claim millions of lives, remaining among the leading causes of death worldwide. In the last years, several efforts have been devoted to shortening and improving treatment outcomes, and to overcoming the increasing resistance phenomenon. Nature has always provided a virtually unlimited source of bioactive molecules, which have inspired the development of new drugs. NPs are characterized by an exceptional chemical and structural diversity, the result of millennia of evolutionary responses to various stimuli. Thanks to their favorable structural features and their enzymatic origin, they are naturally prone to bind proteins and exhibit bioactivities. Furthermore, their worldwide distribution and ease of accessibility has contributed to promote investigations on their activity. Overall, these characteristics make NPs excellent models for the design of novel therapeutics. This review offers a critical and comprehensive overview of the most promising NPs, isolated from plants, fungi, marine species, and bacteria, endowed with inhibitory properties against traditional and emerging mycobacterial enzymatic targets. A selection of 86 compounds is here discussed, with a special emphasis on their biological activity, structure–activity relationships, and mechanism of action. Our study corroborates the antimycobacterial potential of NPs, substantiating their relevance in future drug discovery and development efforts.

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Abbreviations

ADEP	Acyldepsipeptide
AHAS	Acetohydroxyacid synthase
Akt	Alternative name for Protein kinase B
ATP	Adenosine triphosphate
BioB	Biotin synthase
CA	Carbonic anhydrase
Clp	Caseinolytic protease
ClpC	Caseinolytic protease regulatory subunit
ClpP	Caseinolytic protease proteolytic subunit
ClpX	Caseinolytic protease ATP-binding subunit
CoA	Coenzyme A
CoAt	CoA transferase
COVID-19	Coronavirus disease 19
CymA	Cyclomarin A
DAH7P	3-Deoxy-D-arabino-heptulosonate 7-phosphate synthase
DHFR	Dihydrofolate reductase
DprE1	Decaprenylphosphoryl-β-D-ribose oxidase
DSF	Differential scanning fluorimetry
DTB	Dethiobiotin
dTMP	Deoxythymidine monophosphate
dUMP	Deoxyuridine monophosphate
Dxr	1-Deoxy-D-xylulose 5-phosphate reductoisomerase
FAD	Flavin adenine dinucleotide
FAS	Fatty acid synthase
FtsZ	Filamenting temperature-sensitive mutant Z
GlcN-1-P	Glucosamine-1-phosphate
GlmU	*N*-Acetylglucosamine-1-phosphate uridyltransferase
Gyr	DNA gyrase
IC_{50}	Half-maximal inhibitory concentration
IL-6	Interleukin-6
INH	Isoniazid
InhA	Enoyl-acyl carrier protein reductase
KasA	3-Oxoacyl-[acyl-carrier-protein] synthase 1
KasB	3-Oxoacyl-[acyl-carrier-protein] synthase 2
KatG	Catalase-peroxidase
K_d	Dissociation constant
K_i	Inhibition constant
LYP	Lymphoid-specific tyrosine phosphatase
MBC	Minimum bactericidal concentration
MDR-TB	Multidrug-resistant tuberculosis
MetAP	Methionine aminopeptidase
MIC	Minimum inhibitory concentration
MptpA	*Mycobacterium tuberculosis* low-molecular-weight phosphatase A
MptpB	*Mycobacterium tuberculosis* low-molecular-weight phosphatase A
MraY	Phospho-*N*-acetylmuramoyl-pentapeptide-transferase
MRC-5	Medical Research Council cell strain 5 (human lung fibroplast)
Mtb	*Mycobacterium tuberculosis*
MurE	UDP-*N*-acetylmuramoyl-L-alanyl-D-glutamate-2,6-diaminopimelate ligase
MurI	Glutamate racemase
Myx B	Myxopyronin B
NAD	Nicotinamide adenine dinucleotide
NMP	Nonmevalonate pathway
PknG	Protein kinase G
NP	Natural product
NTD	N-terminal domain
PCR	Polymerase chain reaction
PD	Pharmacodynamics
PDB	Protein Data Bank
PET-CT	Positron Emission Tomography-Computed Tomography
PK	Pharmacokinetics
PknG	Serine/threonine-protein kinase G
PPTP1B	Protein phosphotyrosine phosphatase 1B
Psk13	Polyketide synthase
PTP	Protein tyrosine phosphatase
PTP-PEST	Tyrosine-protein phosphatase non-receptor type 12
RIF	Rifampicin
RNAP	RNA polymerase
RR-TB	Rifampicin-resistant tuberculosis
RUF	Rufomycin
SAM	*S*-adenosyl-L-methionine
SAR	Structure-activity relationships
SI	Selectivity index
SK	Shikimate kinase
SUF	Sulfur mobilization machinery
TB	Tuberculosis
TBNAT	*Mycobacterium tuberculosis* arylamine *N*-acetyltransferase
THP-1	Human monocytic leukemia THP-1 cell line
ThyX	Flavin-dependent thymidylate synthase
UDP	Uridine diphosphate
UGM	Uridine 5′-diphosphate galactopyranose mutase
WHO	World Health Organization
WT	Wild type
XDR-TB	Extensively drug-resistant tuberculosis

1. Introduction

Nowadays, tuberculosis (TB), the disease caused by *Mycobacterium tuberculosis* (*Mtb*), is still among the leading causes of mortality from a single infectious agent, possibly surpassed only by COVID-19. In 1993, the World Health Organization (WHO) declared TB a global health emergency; today, after nearly three decades, this disease remains a serious threat, as confirmed by the latest TB Report published last October [1]. The limitations of the available TB drugs represent a major factor underlying the ongoing TB crisis. The current treatment regimen is composed of a cocktail of multiple first-line drugs administered for six months [2]. Despite constituting the best available therapy against TB, these drugs are far from being "ideal". Aside from the toxicity and the duration of the treatment, concerns have been recently raised about their effectiveness. In this regard, Malherbe et al. reported the detection by PET-CT imaging of mycobacterial RNA within non-resolving granulomas in patients who had been declared cured [3]. This case is emblematic of the increasing inadequacy of the existing drug regimens in effectively eradicating persisting *Mtb* bacilli sequestered within granulomas. However, the most serious issue of the available treatment strategies is related to the worrisome spread of resistant *Mtb* strains. In 2019, there were an estimated 465,000 incident cases of multidrug- and rifampicin-resistant TB (MDR/RR-TB), corresponding to 3.3% of all new cases and 17.7% of relapse cases [1]. Most of the resistant strains were insensitive to both rifampicin (RIF) and isoniazid (INH) (78%); even more worryingly, 20% of MDR-TB cases were resistant to

fluoroquinolones, which are commonly used as second-line agents (XDR-TB) [1]. In this dire context, the COVID-19 pandemic is threatening to undo the progresses made over the last years, hindering the detection of TB and causing severe co-infections in fragile patients [1]. Therefore, there is an urgent need for new drugs acting on innovative molecular targets.

Although many anti-TB drug candidates in clinical development are synthetic, most of the approved therapeutic strategies currently used in TB treatment are made up of nature-derived molecules. Natural products (NPs) possess enormous structural and chemical diversity and have profoundly impacted drug discovery and pharmacotherapy. Over the years, many NPs have been found to exhibit promising antitubercular activities [4–9]. However, for many of them a specific target has never been clearly identified, preventing the definition of their mode of action, and, consequently, their development into more advanced clinical candidates.

Considering the increasing difficulty of modern-day drugs to effectively tackle TB, many researchers are directing their efforts towards the rediscovery of traditional medicine and phytotherapy, to explore the possibility of identifying novel anti-TB compounds.

Here, we report a comprehensive update on the latest antitubercular NPs (2010-present) specifically acting on known and validated target enzymes of *Mtb*. All selected compounds were classified in four groups (**A**-**D**) based on their molecular target. For each molecule, we described the source of origin (plants, bacteria, fungi, marine organisms, *etc.*), emphasized the mode of action, and discussed viable opportunities for future improvements. This valuable compendium will hopefully prove useful in providing inspiration for the design and development of new antitubercular agents.

2. Enzyme inhibitors

The selected NPs (2010-present) exhibiting anti-TB properties and acting on specific *Mtb* enzymes were classified in the following four groups, based on the function of their target: (**A**) DNA, RNA, and protein synthesis and metabolism; (**B**) cell wall and fatty acid biosynthesis; (**C**) immune escape mechanisms; (**D**) metabolic pathways. The choice of excluding NPs with unexplored mechanisms of action is motivated by the intent to privilege only the most promising anti-TB candidates. The knowledge of the enzymatic target is fundamental to predict potential off-target interactions with human counterparts and evaluate the likelihood of possible transfers of resistance factors among bacteria. The summary presented in Table 1 contains the main information of several promising leads belonging to the four sets, selected based on their antimycobacterial activity and the comprehensiveness of the biochemical investigations.

2.1. NPs interfering with DNA, RNA, and protein synthesis and metabolism (A)

One of the most successful strategies for the development of antimycobacterial agents is to target key enzymes involved in the biosynthesis and metabolism of nucleic acids and proteins. Among the potential druggable targets, anti-dihydrofolate reductase (DHFR), DNA gyrase (Gyr), and flavin-dependent thymidylate synthase (ThyX) have emerged as the most promising options. Moreover, several NPs have been demonstrated to interfere with RNA polymerase (RNAP), and with enzymes involved in the synthesis and metabolism of proteins, including members of the ClpP protease complex, and constituents of the shikimate, amino acid, and pantothenic acid biosynthetic pathways. The chemical structures of the selected NPs belonging to this class are shown in Figs. 1, 2, 5 and 6.

Folate biosynthesis offers many promising therapeutic targets for anti-TB therapy [10]; remarkable results in the discovery of new NPs against this biochemical route were obtained by Raju and coworkers in 2015 [11]. Starting from the structural similarity of a series of plant polyphenols to antifolate drugs, they explored the inhibition of DHFR by using an *in-silico* approach. Due to its pivotal role in nucleic acid biosynthesis, and its high conservation through evolution, DHFR has served as an ideal antiproliferative drug target for infectious diseases. Based on molecular docking scores of various classes, seven promising polyphenols were identified, with

Table 1
Numerically ordered selection of natural inhibitors with their target, the main biological data, and references.

	Compound	Target(s)	Biological data	Reference(s)
A	**2**	DHFR	$IC_{50} \approx 11$ μM MIC ≈ 4 μM	Raju et al. [11]
	6	ThyX	$IC_{50} \approx 9$ μM MIC ≈ 4 μg/mL (≈21.3 μM)	Sarkar et al. [14]
	8	RNAP	$IC_{50} \approx 0.1$ μM MIC ≈ 1.6 μg/mL (≈3.7 μM)	Srivastava et al. [22] Ebright et al. [23]
	16	Clp protease	$K_d \approx 0.6$ μM $MIC_{90} \approx 160$ nM	Gao et al. [32]
	24	DAH7Ps	$IC_{50} \approx 21$ μM MIC ≈ 10 μg/mL (≈23.2 μM)	Nirmal et al. [46]
B	**30**	MurE	$IC_{50} = 57$ μM MIC = 4 mg/L (≈10.6 μM)	Guzman et al. [54]
	37	UGM TBNAT	UGM at 98.2% and TBNAT 99.1% MIC = 4.1 μM	Šudomová et al. [59]
	40	GlmU	$IC_{50} \approx 14$ μM MIC = 6.25 μg/mL (≈18.6 μM)	Han et al. [63]
	42	KasB	$IC_{50} \approx 2$ μg/mL MIC ≈ 2 μg/mL (*Mtb* H37Rv) (≈4.7 μM)	Wang et al. [67] Moustafa et al. [68]
	43	InhA	$K_i \approx 6$ μM MIC ≈ 0.6 μg/mL (*Mtb* H37Rv) (≈1 μM)	Hartkoorn et al. [72]
	47	BioB	$K_i \approx 1$ μM MIC ≈ 0.6 μM	Bockman et al. [85]
C	**66**	MptpB	$IC_{50} \approx 1.03$ μM MIC 12.3 mg/L (≈32.5 μM)	Chen et al. [101]
D	**81**	CA Rv3273 CA Rv1284	$K_i \approx 9$ μM $K_i \approx 1$ μM MIC ≈ 12.5–50 μg/mL MDR and H37Rv strains (≈37.8–151.3 μM)	Davis et al. [112] Clemente-Soto et al. [113]

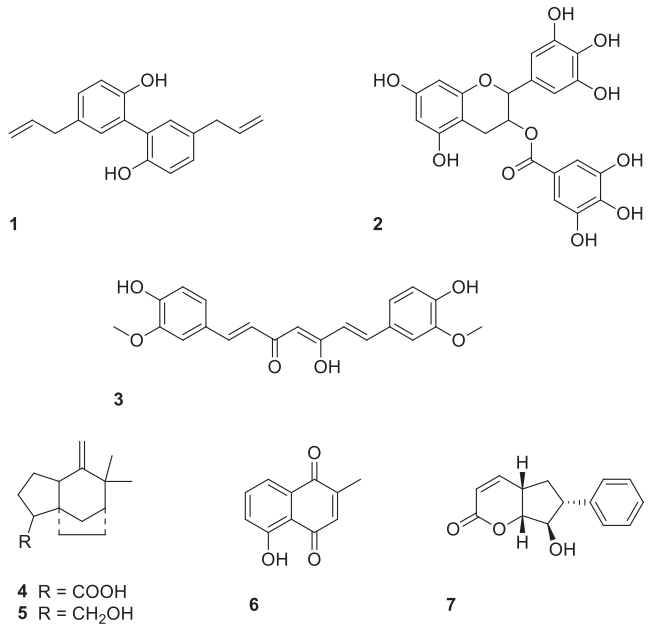

Fig. 1. Chemical structure of inhibitors targeting enzymes involved in DNA biosynthesis.

magnolol (**1**), epigallocatechin gallate (**2**), and curcumin (**3**) showing the best *in vitro* results (IC_{50} values *vs* DHFR: 15.8, 10.9, and 14.6 μM for **1**, **2** and **3**, respectively). When tested against *Mtb* H37Rv in whole-cell assays, they exhibited MIC values ranging from 3.6 to 5.3 μM. These polyphenols were also tested against human DHFR, showing a good selectivity ratio. This is particularly relevant considering that DHFR plays a prominent role in mammals and is the molecular target of established antitumoral drugs, like methotrexate [11].

In 2017, Dwivedi et al. isolated two bioactive constituents from *Vetiveria zizanoides* roots, khusenic acid (**4**) and khusimol (**5**), showing MIC values of about 12 and 25 μg/mL, respectively, when tested against *Mtb* [12]. Docking analyses suggested that **4** and **5** efficiently bound to both the subunits of Gyr (A and B), an essential enzyme involved in DNA replication, transcription, and recombination [13]. These compounds showed good intestinal absorption, aqueous solubility, and proved to be safe and non-toxic [12]; hence, these characteristics make **4** and **5** perfect candidates for the development of new drugs.

Very recently, Sarkar and co-workers found out that plumbagin (**6**) interferes with *Mtb* growth *in vitro* by inhibiting ThyX ($IC_{50} \approx 9$ μM, $K_i = 8.2$ μM) [14]. This essential enzyme uses the reduced form of FAD to deliver reducing equivalents to dUMP, transferring, at the same time, a methylene group from methylenetetrahydrofolate to dUMP, and resulting in the formation of dTMP [15]. Notably, humans and most eukaryotes lack this enzyme, making ThyX a drug target of exceptional importance. Compound **6** is a 1,4-naphthoquinone extracted from plants belonging to the *Plumbaginaceae* family, a source of significant medicinal value. The authors investigated its mechanism of action, demonstrating that **6** inhibits mycobacterial growth (MIC = 4 μg/mL) in a dose-dependent manner [14]. Being a hydroxynaphthoquinone, **6** has the potential to chelate divalent metal ions and form complexes [16]. However, considering that ThyX is not a metalloenzyme, this mechanism is not likely to play a significant role in its inhibitory activity. Further substantiating this point is the fact that lawsone, a close analogue of **6** sharing the hydroxynaphthoquinone scaffold, is inactive against ThyX.

Because iron is an essential cofactor for DNA biosynthesis, natural inhibitors of enzymes involved in metal acquisition may find interesting applications in anti-TB drug development [17–20]. Recently, Elnaas and co-workers studied the binding of altholactone (**7**), derived from the plant *Polyalthia* sp, to Rv1466, a mycobacterial protein involved in the [Fe–S] cluster assembly and

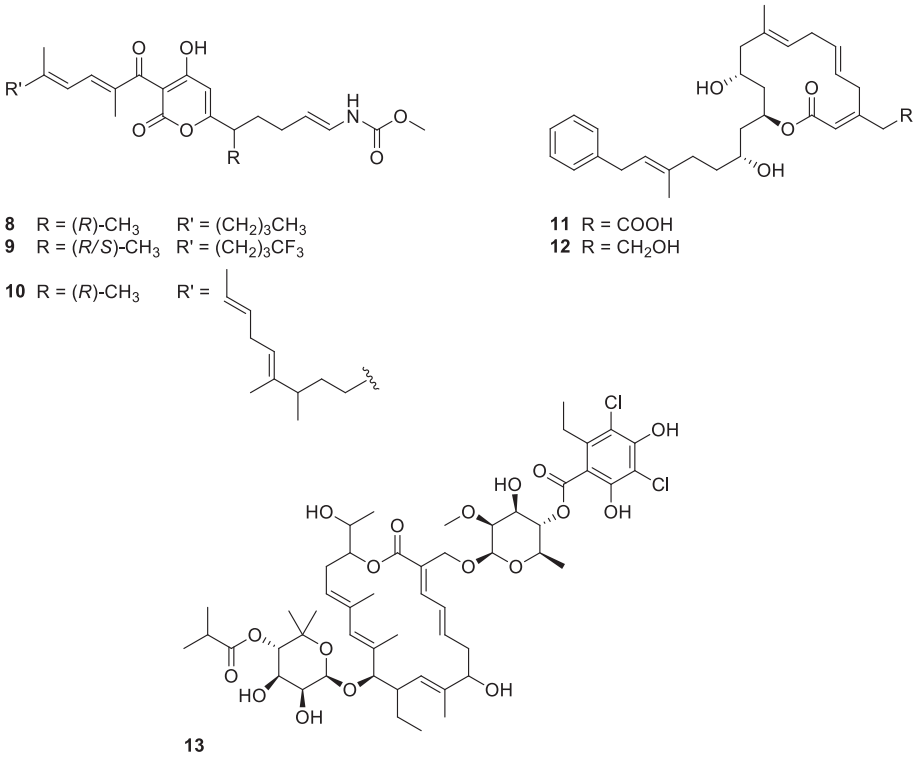

Fig. 2. Chemical structure of inhibitors targeting enzymes involved in RNA biosynthesis.

repair SUF machinery [21]. A pseudo-K_d of 42.0 μM was estimated, and a MIC value of about 27 μM was detected against the H37Ra *Mtb* strain [21]. Further studies are expected shortly, including the use of Rv1466 knock-out strains to confirm the mechanism of action. These outcomes will be pivotal to validate this enzyme and the whole SUF machinery as new emerging targets in anti-TB drug discovery.

Since RR-TB infections represent an important global health problem, the identification of new antibacterial agents inhibiting RNAP is a crucial issue. In this context, the most interesting NP was reported by Srivastava and co-workers: myxopyronin B (Myx B) (**8**), an antibiotic produced by *Myxococcus fulvus* Mf50, exhibited potent antimycobacterial activity against drug-sensitive and drug-resistant strains (MIC against *Mtb* H37Rv = 1.6 μg/mL) [22]. Compound **8** showed no cross-resistance with RIF and, when co-administered with this first-line agent, it exhibited a synergistic antibacterial activity, suggesting a different mode of action. Two mutually non-exclusive models have been proposed to explain the activity of **8**. According to the first theory, **8** is capable of hindering conformational changes in the RNAP switch region, preventing the opening of the enzyme clamp and the loading of the DNA into the active-site cleft. A second hypothesis holds that **8** interferes with contacts between the switch region and the unwound DNA template strand required for DNA unwinding [22]. Hence, despite **8** possesses structural features that may suggest an ability to bind to metal ions, it does not interact with the Mg^{2+} of the active site of RNAP, but rather to a different region [22]. On these bases, Ebright et al. synthesized several derivatives of **8** to obtain more potent RNAP inhibitors; among them, compound **9** showed the highest inhibitory activity (IC_{50} = 30 nM *vs* **8** IC_{50} = 100 nM) [23]. Another effective antibiotic structurally related to **8** is corallopyronin (**10**), an α-pyrone produced by *Corallococcus coralloides* Cc c127, which displayed a MIC value of 3.1 μg/mL. For this compound, the theorized mechanism of action was again related to its capacity to provoke conformational changes of RNAP [22]. An inhibitor of bacterial RNAP mechanistically distinct from rifamycin-derived inhibitors is ripostatin B (**11**), a natural polyketide-derived 14-membered macrolide of myxobacterial origin (*Sorangium cellulosum* So ce377) [24]. Recently, Glaus F. et al. synthesized a series of new ripostatin analogues, which showed good activity against *Mtb* RNAP in a promoter non-specific transcription assay; among them, derivative **12** showed the best IC_{50} value (0.4 μM *vs* 2.8 μM of **11**) [25]. In the above-mentioned paper, Srivastava and co-workers also described the isolation and biological characterization of lipiarmycin (**13**), an 18-membered macrocyclic-lactone antibiotic produced by different species of the Actinomycete family (e.g., *Actinoplanes deccanensis*, *Micromonospora echinospora*, and *Dactylosporangium aurantiacum hamdenensi*); the compound exhibited a MIC value of 3.1 μg/mL against *Mtb* H37Rv [22].

Members of the Clp family of ATP-dependent protease complexes constitute a primary protein degradation system in *Mtb*. The Clp protease is essential for pathogen viability; this enzyme is composed of a proteolytic subunit (ClpP) and a regulatory ATPase subunit (ClpC1 or ClpX). In detail, *Mtb* possesses two co-transcribed genes (*clpP1* and *clpP2*) whose products form discrete heptameric rings that associate to form the active heterotetradecamer ClpP1P2, stabilized by interaction with ClpX or ClpC1 [26]. Notably, ClpX is involved in the regulation of the cell division cycle: it interacts with the filamenting temperature-sensitive mutant Z (FtsZ), a homologue of the eukaryotic tubulin, responsible for the formation of the septum at the site of division (Z-ring) [27]. Despite FtsZ is not an enzyme, it occupies a prominent position among emerging anti-TB targets. In the last years, successful studies have led to the discovery of several FtsZ inhibitors, many of which are derived from natural sources [28].

In general, the antimycobacterial effect of Clp ligands may be due either to the accumulation of toxic undegraded proteins or to the enhanced breakdown of essential biomolecules. Hence, the identification of new NPs targeting the components of the Clp protease complexes is of special interest [29]. Acyldepsipeptides (ADEPs) are a class of bacteria-derived antibiotics, which act by deregulating the ClpP protease. Natural ADEPs were originally identified as products of *Streptomyces hawaiiensis* NRRL 15010 by Thomy et al. [30]. Schmitz and co-workers observed that **14** inhibits the growth of *Mtb* H37Rv with a MIC of 50 μg/mL; its fragment **15** showed a MIC of 12.5 μg/mL against the same strain. While the lethality of ADEPs (like **14**) was found to be linked to the inhibition of essential proteolytic activities, fragment **15** unexpectedly enhanced the rate of the ATP-dependent degradation of protein substrates by the ClpXP1P2 assembly [31]. In detail, **14** and **15** stimulated ClpP1P2 peptidase activity with EC_{50} of 46 μM and 38 μM, respectively, but showed different effects on ClpXP1P2-catalyzed proteolysis: while **15** enhanced ClpXP1P2 with an EC_{50} of 40 μM, **14** inhibited it with an IC_{50} of 59 μM [31].

Gao et al. screened more than 65,000 actinomycete extracts for potential inhibitory activity against the Clp complex. These studies brought to the identification of ecumicin (**16**), a macrocyclic tridecapeptide isolated from the actinobacterium *Nonomuraea* sp. MJM5123. Compound **16** blocks ClpC1-mediated protein breakdown but stimulates the hydrolysis of ATP at submicromolar concentrations (K_d = 0.6 μM). This activity was demonstrated to be selective over mammalian cells, with a selectivity index (SI) of 640 [32]. Very recently, Wolf et al. reported the co-crystal structure of **16** bound to the ClpC1 N-terminal domain (ClpC1-NTD), which allowed to derive its mechanism of action (Fig. 3) [33]. Compound **16** displayed a potent bactericidal activity (*Mtb* H37Rv MIC_{90} = 160 nM), dependent upon both the concentration and time of exposure; the activity was maintained against several mono-resistant strains. To overcome its poor water solubility, a polymeric micelle formulation was developed for parenteral administration in mice, obtaining a complete inhibition of the growth of *Mtb* in the infected lungs after 12 doses at 20 mg/kg [32].

Another NP targeting ClpC1-NTD is the cyclic depsipeptide ohmyungsamycin A (**17**), isolated from the marine actinobacterium *Streptomyces* sp. SNJ042 and studied against *Mtb* (MIC_{90} = 110 nM)

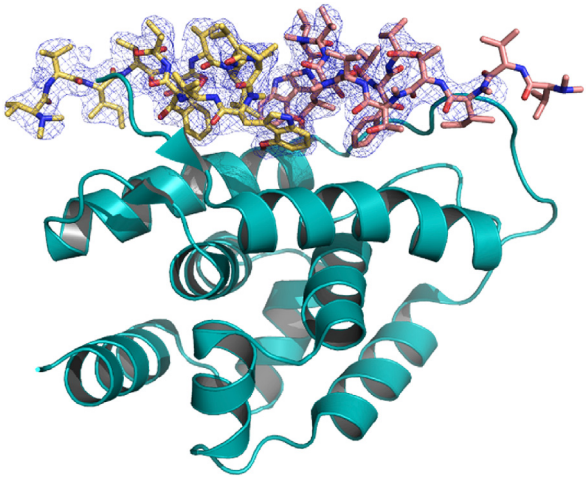

Fig. 3. Ribbon diagram of the monomeric structure of the ClpC1-NTD-**16** complex (PDB code: 6PBS), evidencing the 1:2 stoichiometry. The two molecules are represented in sticks, and the blue mesh represents the electron density around the ligands (contoured at 1σ). **16** has an extended tail of three amino acids that protrudes from the structure of the complex, playing a significant role in the binding of the N-terminus of ClpC1-NTD. **16** enhances the ATPase activity of ClpC1 because, upon ligand binding, a conformational modification increases the accessibility of ATP to its binding pocket.

by Kim and co-workers [34]. Like **16**, **17** increased proteolysis, probably by acting on ClpC1, and induced cell death through unregulated protein degradation. A preliminary work by Hawkins et al. provided the basis for further SAR studies on synthetic analogues by proposing a robust solid-phase route [35]. Starting from a whole-cell screening of NPs, Schmitt et al. identified cyclomarin A (CymA) (**18**), an antibiotic heptapeptide from *Streptomyces* sp. CNB-982, which showed potent activity against *Mtb* with a MIC_{50} of 0.1 μM. Compound **18** bound to ClpC1-NTD with high affinity, and inducing increased proteolysis and cell death [36]. Structures of the wild-type (WT) and mutant ClpC1-NTD from *Mtb* were solved by X-ray crystallography either in the absence or in the presence of **18**. From these data, the authors hypothesized that the binding of **18** reduced the flexibility of the linker between the two N-terminal repeats, making them immobile and leaving the ClpC1 tunnel open. This would allow free access to larger proteins into the proteolytic core of the Clp complex, leading to the degradation of functional or partially folded nascent proteins [37,38].

ClpC1 is also the molecular target of rufomycins (RUFs), a family of potent anti-TB cyclic heptapeptides isolated from the *Streptomyces atratus* strain MJM3502 [39]. These NPs significantly decreased the proteolytic capabilities of the protease complex to degrade casein, while having no significant effect on the ATPase activity of ClpC1. This mechanism represents a marked difference from **16**, which inhibits ClpC1 proteolysis, but stimulates the ATPase activity. Hence, although these peptides share ClpC1 as their macromolecular target, their downstream effects are distinct, likely due to differences in binding. Zhou and co-workers identified rufomycin I (**19**) as a potent and selective lead for *Mtb* (MIC = 0.02 μM). The X-ray structure of the ClpC1-NTD-**19** complex (PDB entry code: 6CN8; $K_d \approx 100$ nM) revealed distinct differences to the previously reported co-crystal of ClpC1 with **16** (Fig. 4). Conversely, **18** and **19** displayed a similar binding mode; however, differently from **18**, the epoxide moiety of **19** opened and covalently bound to ClpC1-NTD *via* the sulfur atom of Met1. The slower formation of the covalent adduct is consistent with the long-term bactericidal effects of this class of cyclic antibiotics [39]. Notably, **19** offers a lower-molecular-weight alternative to **16**, potentially avoiding solubility and cellular penetration issues. Moreover, **19** maintained its activity against MDR and XDR strains of *Mtb*. SAR studies on analogues of **19** indicated that their MIC values varied greatly with small structural changes; in particular, the epoxy group of **19** was identified as a critical moiety for both the enzymatic binding and the bactericidal activity. Semisynthetic modifications of RUFs are ongoing, together with pharmacokinetic/pharmacodynamic (PK/PD) studies on this class of potential anti-TB leads [39].

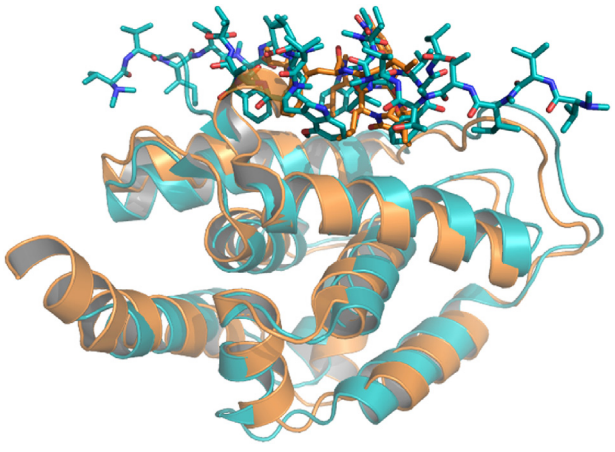

Fig. 4. Superposition of the ClpC1-NTD-**16** (PDB code: 6PBS) and ClpC1-NTD-**19** (PDB code: 6CN8) complexes. The former is represented in teal, while the latter is in orange. The image evidences the different binding modes of **16** and **19**, as well as the conformational adaptations of the protein, which were linked to the different mode of action of the two compounds. Differently from **16**, **19** showed no significant effect on the ATPase activity of ClpC1. Likewise, the structurally related compound **18** proved to have no effect on the ATPase activity, displaying a similar binding mode to **19** (but an opposite effect on proteolysis).

Components of plant essential oils have been recently found to interfere with Clp proteases. Sawicki et al. determined the MIC value of cinnamaldehyde (**20**), the main constituent of cinnamon essential oil, against *Mtb* H37Ra ATCC 25177 strain (8 μg/mL). To determine whether **20** and the essential oil had a bacteriostatic or bactericidal effect, the MBC was determined. Both samples revealed an MBC equal to 32 μg/mL, but **20** had an SI of 14.08, showing a significantly less cytotoxic effect than cinnamon essential oil (SI = 8.7) [40]. The transcriptional profiling revealed the overexpression of the *clgR* gene, which encodes for a regulator that controls the ClpP protease [29,41], activated during *Mtb* growth within macrophages. Compound **20** probably threatens the membrane integrity and activates the stress response system [40]. Analogously, two terpenes commonly occurring in essential oils, β-elemene (**21**) and *R*-limonene (**22**), significantly altered the expression of the *clgR* gene. Moreover, **21** and **22** influenced the expression of DprE1, another important marker of the integrity and stress status of the envelope; both exhibited MIC values of 32 μg/mL [42].

Despite ClpC1 is currently not targeted by any of the approved anti-TB treatments, all these results support its viability as a target for the design of new drugs.

In mycobacteria, the synthesis of proteins relies on essential enzymes, which include methionine aminopeptidase (MetAP), 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase (DAH7Ps), shikimate kinase (SK), and acetohydroxyacid synthase (AHAS); hence, their fundamental role makes them attractive targets for the development of new antibiotics. MetAP removes the N-terminal methionine residue from newly synthesized proteins [43]. Starting from the scaffold of natural bengamides, first isolated from coral reef sponges, Lu et al. developed a new class of MetAP inhibitors as antitubercular therapeutics. To synthesize different bengamide derivatives, they replaced the caprolactam moiety with various amide moieties, obtaining inhibitors with high potency and selectivity *vs* the human homologue. Derivative **23** exhibited the best antitubercular activity against both replicating (MIC = 50.6 μM) and non-replicating bacteria (MIC = 107.4 μM), together with good inhibitory activities against MetAP1a and MetAP1c (the two MetAPs from *Mtb*), with IC_{50} values of 7.9 and 0.54 μM, respectively [44,45]. Unfortunately, the biological test against human K562 cells indicated a partial activity on mammalian MetAPs [45].

The shikimate pathway plays an essential role in the biosynthesis of the three aromatic amino acids (phenylalanine, tyrosine, and tryptophan) in *Mtb*. Notably, the enzymes involved in this process are absent in humans, which makes them ideal targets for the development of anti-TB agents. Therefore, Nirmal et al. employed a pharmacophore-based virtual screening using synthetic and NP databases to identify new DAH7Ps inhibitors [46]. Two interesting NPs were selected: α-tocopherol (**24**) and the citrus flavonoid glycoside rutin (**25**), which inhibited DAH7Ps with IC_{50} values of 21 μM and 42 μM, respectively [46]. Furthermore, compound **24** showed MIC ≈ 10 μg/mL against *Mtb* H37Rv [47]. In this context, Masoko P. et al. investigated the activity of *Sutherlandia frutescens* extracts against SK, which catalyzes the fourth step of the shikimate pathway: the identified α-linolenic acid (**26**) displayed an IC_{50} value of 3.7 μg/mL against this target [48]. Compound **26** was examined for its antimycobacterial activity against Mtb H37Rv, showing a MIC ≈ 75 μg/mL [49]. Despite the apparently promising results, further efforts are needed to determine whether **24**, **25**, and **26** are capable to arrest the growth of *Mtb* bacilli.

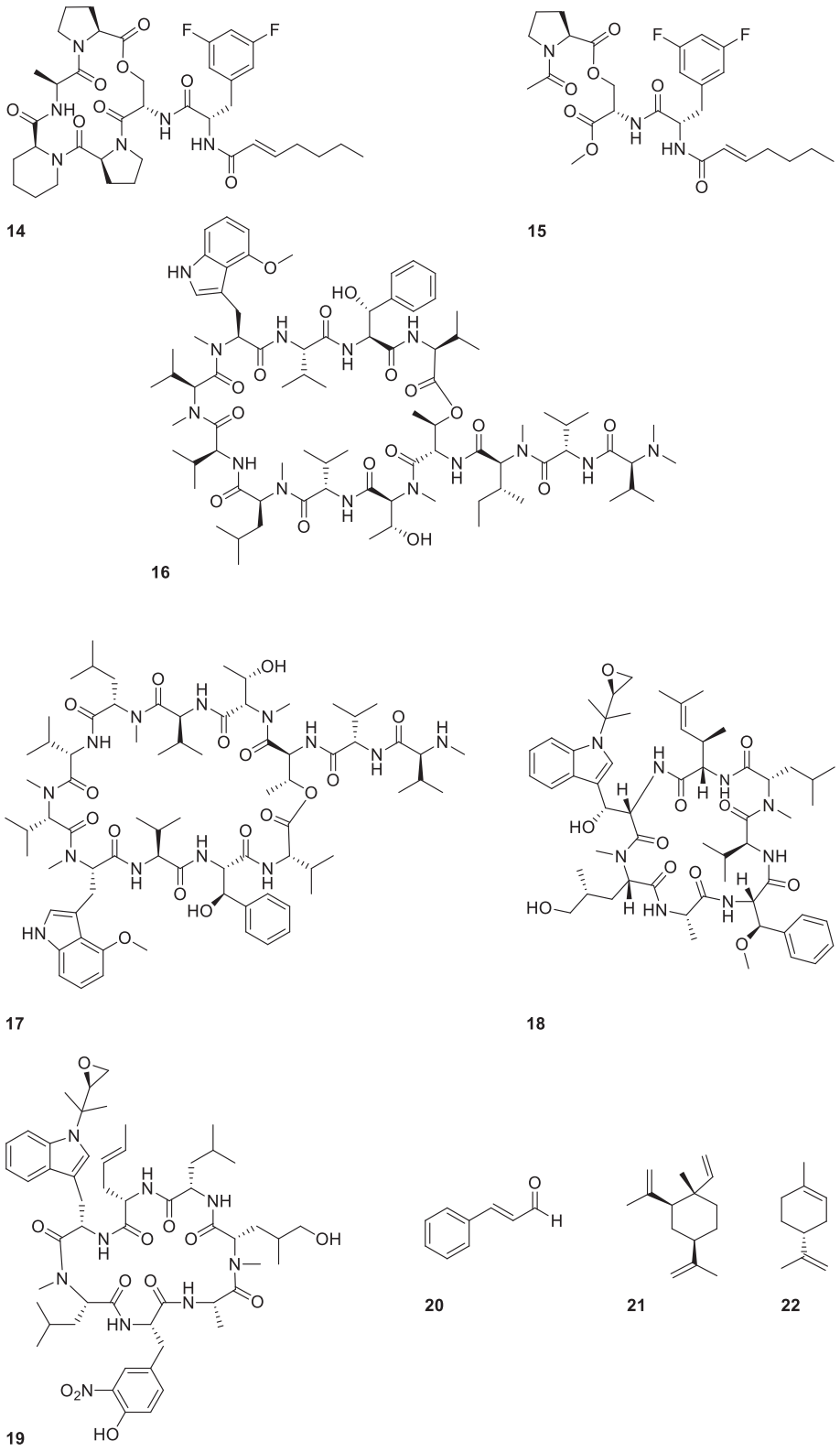

Fig. 5. Chemical structure of inhibitors targeting the Clp complex.

AHAS, involved in the biosynthesis of branched-chain amino acids and pantothenic acid, was studied by Rehberg and co-workers as the target of chlorflavonin (**27**), a flavonoid from the endophytic fungus *Mucor irregularis*, obtained from the medicinal plant *Moringa stenopetala*. Compound **27** exhibited a potent growth inhibitory effect *in vitro* against *Mtb*, with a MIC_{90} value of 1.6 μM; importantly, cytotoxicity assays showed no activity against human cell lines MRC-5 and THP-1 up to concentrations of 100 μM. Mapping of resistance-mediating mutations, employing whole-genome sequencing, chemical supplementation assays, and molecular

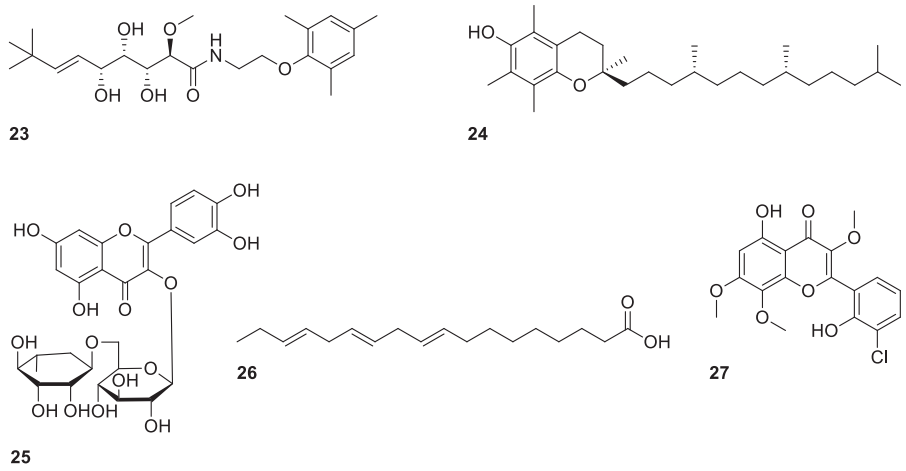

Fig. 6. Chemical structure of inhibitors targeting MetAP, DAH7Ps, SK, and AHAS.

docking studies confirmed that **27** acts as a selective inhibitor of AHAS [50].

2.2. NPs interfering with cell wall and fatty acid biosynthesis (B)

The cell wall of mycobacteria shares some features with that of other bacteria, but shows several key biochemical and structural differences, which make it unique among prokaryotes. This complex structure is composed of three layers (peptidoglycan, arabinogalactan, and mycolic acids) and is essential for cell growth and virulence. The fundamental role and peculiarity of the mycobacterial cell wall has prompted the exploitation of the pathways involved in its biosynthesis and assembly for the development of antitubercular drugs [51].

Important classes of antimycobacterial agents act by interfering with the synthesis of the cell wall, weakening the peptidoglycan scaffold to the point that its structural integrity eventually fails. Since mammalian cells have a plasma membrane but lack the peptidoglycan layer, the biosynthetic pathways leading to the formation of this structure constitute attractive targets for innovative anti-TB agents. NP inhibitors of glutamate racemase (MurI), UDP-*N*-acetylmuramoyl-L-alanyl-D-glutamate-2,6-diaminopimelate ligase (MurE), phospho-*N*-acetylmuramoyl-pentapeptide-transferase (MraY), uridine 5′-diphosphate galactopyranose mutase (UGM), and *N*-acetylglucosamine-1-phosphate uridyltransferase (GlmU) showed promising anti-TB activities. The chemical structures of the selected NPs belonging to this class are illustrated in Figs. 7, 8 and 10.

Pawar and co-workers screened various classes of NPs as potential inhibitors of MurI using a computational approach [53]. MurI catalyzes the racemization of *l*-glutamate to *d*-glutamate, a process that is involved in the biosynthesis of peptidoglycan [52]. Naringenin (**28**) and quercetin (**29**) showed the highest binding scores among the selected compounds, and kinetic studies indicated that **28** and **29** competitively inhibited MurI with a K_i of 23.8 μM and 20.8 μM, respectively [53]. The relatively fast-growing and non-pathogenic *M. smegmatis* was used to evaluate their low anti-mycobacterial activity ($MIC_{50} > 200$ μM), but fluorescence and electron microscopy confirmed the appearance of membrane and cell wall damages upon exposure to these flavonoids [53].

Guzman et al. studied several components of Colombian *Lauraceae*, *Magnoliaceae,* and *Piperaceae* species, as sources of potential antimycobacterial metabolites. The most interesting results were obtained for **30**, which showed significant MIC values against both *M. bovis* BCG and *Mtb* H37Rv (≈4 mg/L), attributable to MurE inhibition ($IC_{50} \approx 57$ μM) [54]. The SI of 12 demonstrated the specificity of **30**, and further tests proved the low toxicity against macrophages (not affected up to 50 mg/L) [54].

In a previous study performed by Xie et al., sansanmycins, a class of antibiotics produced by the soil bacterium *Streptomyces* sp. SS, exhibited antibacterial activity against *Mtb* H37Ra [55]. Tran and co-workers synthesized a library of dihydrosansanmycins, and three analogues **31**, **32**, and **33** exhibited excellent inhibitory activities against *Mtb*, with MIC_{50} values of 80, 180, and 37 nM, respectively [9]. Moreover, **31**, **32**, and **33** showed inhibitory properties against MraY, with IC_{50} values of 54, 48, and 41 nM, respectively. Their antimycobacterial activity was assessed in an intracellular assay: they exhibited IC_{50}s of about 1.6, 4.3, and 0.1 μM, respectively. Furthermore, each compound showed excellent stability, with degradation half-lives >7 h for human and mouse plasma, and >160 min for human and mouse liver microsomes [9].

Villaume and co-workers investigated some flavonoids as potential UGM inhibitors, leading to the identification of luteolin (**34**), which exhibited a nearly complete inhibition and good affinity for UGM ($K_d = 34$ μM) [56,57]. Compound **34** was tested for its anti-TB activity, and the results showed a MIC of about 100 μg/mL [57]. **34** was identified as a noncompetitive inhibitor of UGM, displaying a strong affinity for an allosteric site of the enzyme in its opened form [56]. Furthermore, several derivatives of **34** were synthesized and tested: notably, **35** potently inhibited the enzyme, showing a halved MIC value with respect to the parent compound (MIC = 50 μg/mL). SAR studies among this class evidenced the key role played by the flavone scaffold in the inhibition and affinity for UGM. Moreover, the hydroxyl group at the *para* position of the phenyl ring was found to be essential for optimal binding, while modifications to the other aromatic ring did not improve the potency [57].

Psoromic acid (**36**), a β-orcinol depsidone widely present in the lichen species, was tested by Hassan et al. against nine strains of *Mtb*, resulting in MIC values in the range 3.2–4.1 μM (*Mtb* H37Rv MIC = 3.2 μM), and good selectivity indices (SI ≈ 20). Compound **36** inhibited UGM at 85.8% and exerted a significant inhibitory activity also against *Mtb* arylamine N-acetyltransferase (TBNAT) ($IC_{50} = 8.7$ μM), further reducing the mycobacterial cell wall mycolates, without displaying any cytotoxic effects on human liver hepatocellular carcinoma cells [58]. A similar dual mechanism of action against UGM and TBNAT was evidenced by Šudomová and co-workers, who tested fucoxanthin (**37**), a naturally occurring carotenoid. **37** inhibited UGM at 98.2% and TBNAT at 99.1%, displaying MIC values in the range 2.8–4.1 μM (*Mtb* H37Rv

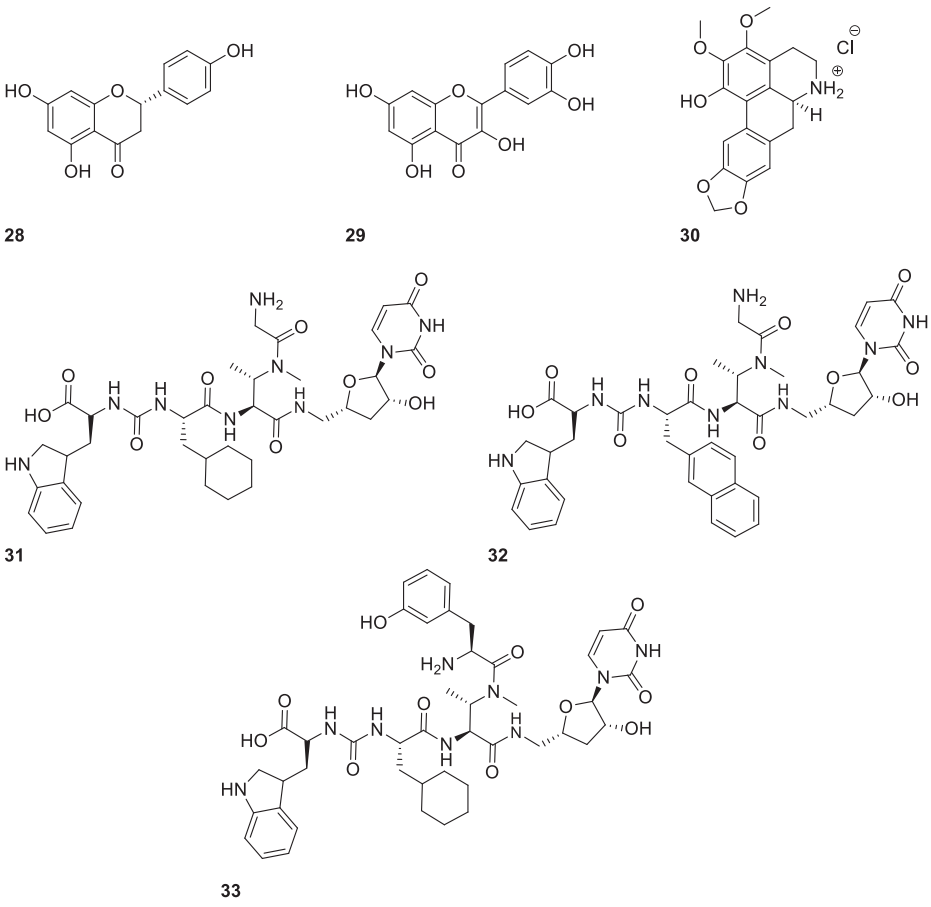

Fig. 7. Chemical structure of inhibitors targeting enzymes involved in cell wall biosynthesis.

MIC = 4.1 μM), with a good degree of SI (ranging from 6.1 to 8.9) [59].

Yu and co-workers isolated several diterpenoids from the roots of *Euphorbia ebracteolata* and characterized their biological activity as inhibitors of the synthesis of lipopolysaccharide and peptidoglycan compounds **38** and **39** showed inhibitory effects against *Mtb*, acting as GlmU inhibitors with IC_{50} values of 12.5 and 41.8 μg/mL, respectively [60,61]. Additionally, **38** showed a good MIC value of 15 μg/mL [61,62].

Han et al. studied dicumarol (**40**), a natural derivative of coumarin, which exhibited growth inhibitory activity against *Mtb* [63]. Its MIC values were calculated against *Mtb* H37Ra, and against strains overexpressing the control vector pVV2 (H37Ra/pVV2) and GlmU (H37Ra/pVV2-glmU). The MIC for *Mtb* H37Ra/pVV2-glmU was higher (12.5 μg/mL) than the MIC of 6.25 μg/mL detected for both the H37Ra and H37Ra/pVV2 mycobacterial strains. Notably, **40** increased the sensitivity of the *Mtb* isolates to first-line anti-TB drugs, suggesting that this synergistic effect may be exploited for the design of new combination therapies with INH or RIF. **40** exhibited inhibitory activity against GlmU, with an IC_{50} value of 13.7 μM; instead of directly blocking GmlU active site, **40** interfered with the GlmU-acetyl CoA complex, affecting the binding of the second substrate, glucosamine-1-phosphate (GlcN-1-P) to the complex [63].

Fatty acid biosynthesis has a significant potential as a target for the development of novel antimycobacterials. Interesting NPs were identified as inhibitors of the ketosynthases of the FAS-II complex, enoyl-acyl carrier protein reductase (InhA), polyketide synthase (Psk13), and biotin synthase (BioB).

Machutta et al. isolated thiolactomycin (**41**), naturally produced by species of *Nocardia* and *Streptomyces*, as a promising lead compound for the inhibition of KasA, one of the two ketosynthases of the FAS-II complex. This compound showed an IC_{50} of 19 μM *vs* KasA and a moderate MIC of 62.5 μM; moreover, it was also active against MDR and XDR-TB strains, albeit at a higher concentration. Notably, its favorable physical properties established a rationale for the development of semisynthetic derivatives [64]. Additionally, the determination of the crystal structure of the KasA-thiolactomycin complex allowed the investigation of its binding mode [65]. All these findings prompted the design of analogues having higher affinity for KasA compared to the parent compound, through modifications at the thiolactone C3 position [66].

Previously, Wang et al. isolated the NP (±)-platencin (**42**) from strains of *Streptomyces platensis*, which showed inhibitory activity against KasB, with an IC_{50} value of 1.95 μg/mL [67]. Notably, **42** exhibited a potent bacteriostatic activity towards *Mtb* H37Rv (MIC = 2 μg/mL) and MDR and XDR resistant strains (MIC = 1 μg/mL) [68]. Because of their unique structures and potent antibacterial activities, several derivatives were prepared through synthetic and semisynthetic approaches [69].

Being the molecular target of the first-line drug INH, the enzyme InhA has occupied a central role in the development of anti-TB drugs for decades. However, INH is a prodrug that needs to be activated by the catalase-peroxidase KatG, a non-essential *Mtb* enzyme. Indeed, different mutations in the *katG* gene, together with mutations in *inhA*, are responsible for the insurgence and spread of several *Mtb* isolates resistant to INH [70]. For this reason, numerous studies have focused on developing new inhibitors.

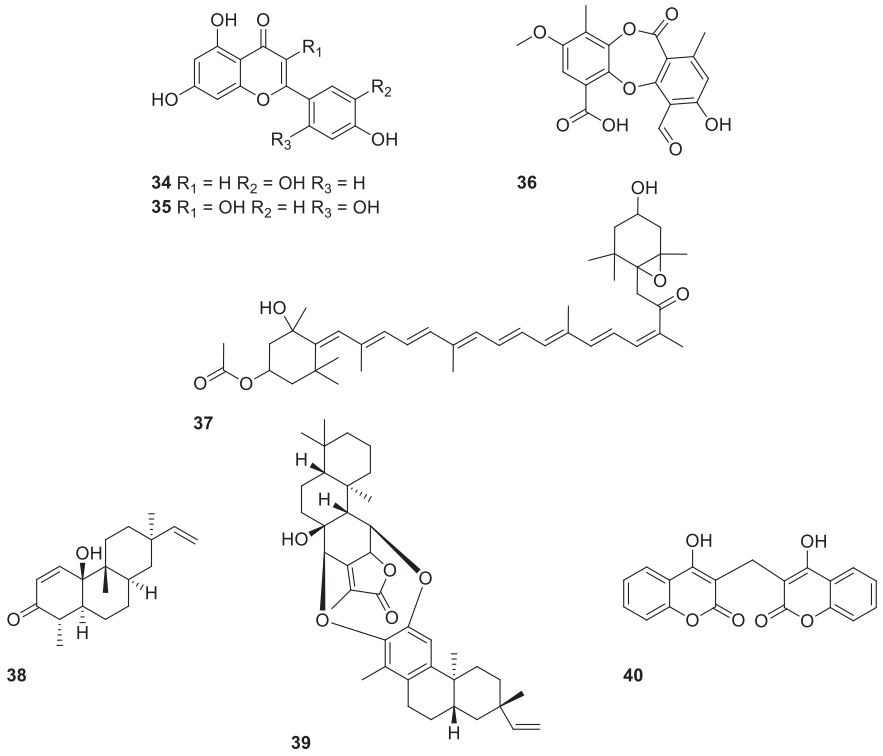

Fig. 8. Chemical structure of inhibitors targeting enzymes involved in the cell wall biosynthesis.

However, many of them suffer from a poor activity against the pathogens. The bacterial secondary metabolite pyridomycin (**43**), produced by *Streptomyces pyridomyceticus* or *Dactylosporangium fulvum* [71], was found to be a direct inhibitor of InhA ($K_i = 6.5$ μM) and exhibited significant *in vitro* antimycobacterial activity against several *Mtb* strains, including H37Rv (MIC = 0.56 μg/mL), and INH-resistant clinical isolates [72]. Crystallographic investigations evidenced that **43** bound to the InhA active site differently from INH, blocking the binding sites of both the NADH cofactor and the lipid substrate [73,74]. Fig. 9 illustrates the interaction of **43** within the wide and deep InhA pocket, which may offer the opportunity to introduce structural modifications on this scaffold to enhance the affinity. These promising results prompted the search of new derivatives of **43**. Recently, Kienle and co-workers synthesized and tested several analogues, which demonstrated the importance of the ester function and of the original configuration of the stereocenters for the activity. Moreover, they identified new dihydropyridomycins having a comparable antimycobacterial activity to the natural lead [75].

A 3D ligand-based virtual screening carried out by Pinzi and co-workers evidenced the structural similarities of cannabigerol (**44**) and cannabichromene (**45**), two cannabinoids from *Cannabis sativa* L., to 5-pentyl-2-phenoxyphenol, which acts against InhA with high efficacy. Interestingly, **44** showed an IC_{50} value of 5.2 μM against InhA, whereas **45** turned out to be scarcely active [76].

A key step of the biosynthesis of mycolate-containing compounds involves Pks13, an enzyme that catalyzes the condensation of long fatty acid derivatives to the meromycolyl chains, leading to α-alkyl β-ketoacids [77]. Moreover, since several and different inhibitors of Pks13 have been identified, this enzyme has been classified as a so-called promiscuous target, which underlines its importance in the drug discovery and development process [78]. Because coumestan constitutes the central core of various bioactive NPs [79,80], Zhang and co-workers synthesized several derivatives as new potential anti-TB agents targeting Pks13 [81,82]. Whole-genome deep sequencing of the WT enzyme and of resistant mutants confirmed that these derivatives inhibited Pks13. The molecular interaction between selected inhibitors and the thioesterase domain of the enzyme (Pks13-TE) was characterized by thermal stability analysis using the nano differential scanning fluorimetry (nanoDSF) method [83]. Derivative **46** showed a thermal stabilization (ΔT_m^a) of about 10 °C and demonstrated excellent anti-TB activity against both drug-susceptible (MIC = 0.0039 μg/mL), and drug-resistant *Mtb* strains (MIC = 0.0078 μg/mL), favorable human microsomal stability, selectivity against normal cells, as well as oral bioavailability in mice. Furthermore, **46** showed an 8-fold higher activity than INH *in vivo* [82].

Recently, the antimicrobial racemic acidomycin (**47**), originally isolated from culture filtrates of *Streptomyces virginiae*, *Streptomyces lavendulae*, *Streptomyces acidomyceticus*, and *Streptomyces cinnamonensis* [84], was reinvestigated as a potential candidate for anti-TB drug development, considering the extremely low frequency of spontaneous resistance to this compound. Bockman et al. evaluated the activity of the acidomycin enantiomers: **(*S*)-47** revealed a MIC of 0.6 μM, while the unnatural enantiomer **(*R*)-47** was less active, with a MIC of 7.7 μM [85]. Deeper studies confirmed that the racemic NP competitively inhibited BioB, an enzyme that catalyzes the conversion of dethiobiotin (DTB) to biotin by the insertion of a sulfur atom into DTB, with a K_i of about 1 μM. The purified **(*S*)-47** was 14-fold more potent than the (*R*) enantiomer. **47** also stimulated unproductive cleavage of *S*-adenosyl-L-methionine (SAM) to generate the toxic metabolite 5′-deoxyadenosine, responsible for the anti-TB activity against a series of drug-susceptible and drug-resistant strains. The SAR study highlighted that the activity of these derivatives was sensitive to minor changes. Additionally, **47** proved to be inactive *in vivo* in mice models, probably because of its poor PK properties, considering that it was rapidly eliminated with a half-life of 14.4 min. Therefore,

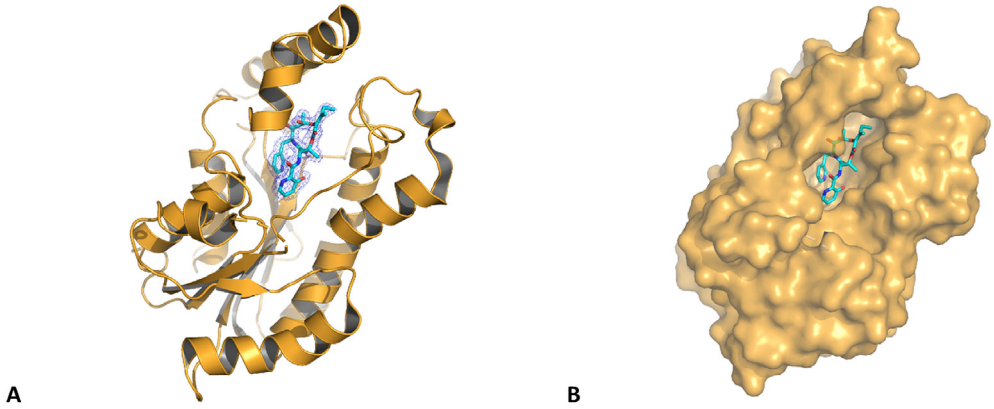

Fig. 9. A. Ribbon diagram of InhA in complex with **43** (PDB code: 4BII). The ligand is represented in sticks; the blue mesh represents the electron density around the compound (contoured at 1σ). **B.** Illustration of the surface of InhA, with **43** in the active site. The ligand occupies a large protein cavity, effectively blocking the access to the binding pockets of both the NADH cofactor and the lipid-substrate.

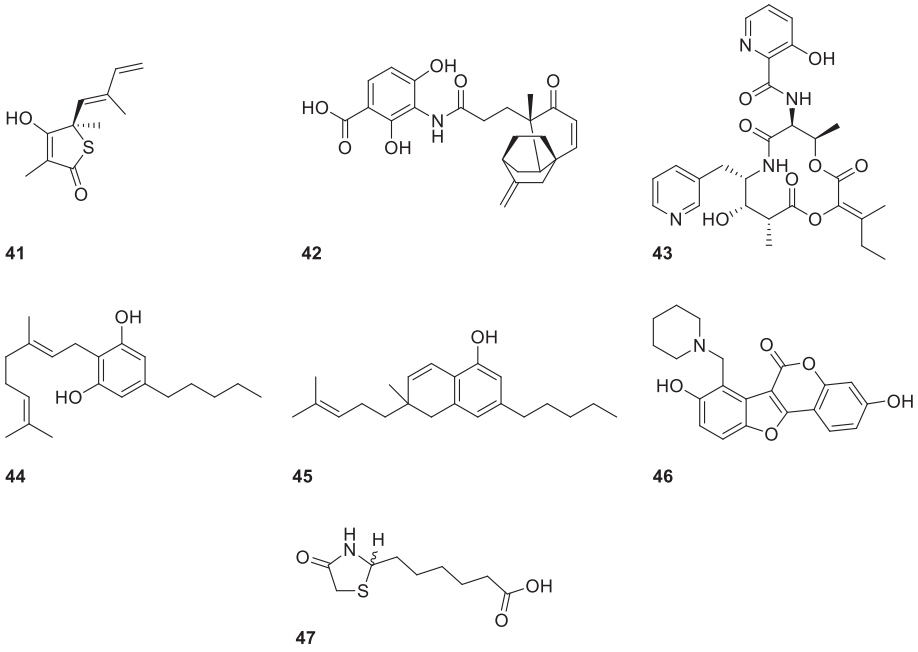

Fig. 10. Chemical structure of inhibitors targeting enzymes involved in fatty acid biosynthesis.

further efforts in this field should be addressed to the design of prodrugs, with the aim of improving the bioavailability [85].

2.3. NPs interfering with immune escape mechanisms (C)

Among the reasons behind the resilience of *Mtb* is its ability to survive and replicate within host macrophages: the mycobacterium exploits several escape mechanisms, specifically evolved to evade the immune system of the host. Hence, these strategies could be conveniently exploited to develop anti-virulence compounds aimed at preventing the attack to the host, rather than killing the pathogens. These novel approaches should reduce the insurgence of resistance mechanisms, as they would not exert a selective pressure on the pathogen [86]. Over the last decade, several NPs capable of preventing the block of the maturation and acidification of lysosomes have been studied. Most of the recently identified natural therapeutics are inhibitors of the low-molecular-weight phosphatases (PTPs) MptpA and MptpB [87], and of the protein kinase G (PknG). The chemical structures of the selected NPs belonging to this class are shown in Figs. 11–13.

Marine-derived bacteria and fungi have been identified as invaluable sources of candidate NPs [88]; in particular, several studies investigated the biological activity of compounds extracted from mangrove endophytic fungi, belonging to the genus *Aspergillus* spp. Liu and co-workers performed a screening of a library of marine-derived NPs to detect new anti-TB agents against MptpA, a phosphatase involved in the inhibition of phagosome-lysosome fusion [89,90]. The MptpA inhibitory activity exhibited by the crude *Aspergillus sydowii* MF357 extract was attributed to sydowiols A (**48**) (IC_{50} = 14 μg/mL) and C (**49**) (IC_{50} = 24 μg/mL) [90]. Using a similar approach, Huang and co-workers identified several NPs capable of inhibiting MptpB from the same natural source. MptpB arrests the maturation of vacuoles and blocks the production of IL-6, promoting host cell survival by activating Akt and inhibiting caspase 3 [91,92]. The most active compound was asperterpenoid A (**50**), which exhibited a very potent inhibitory

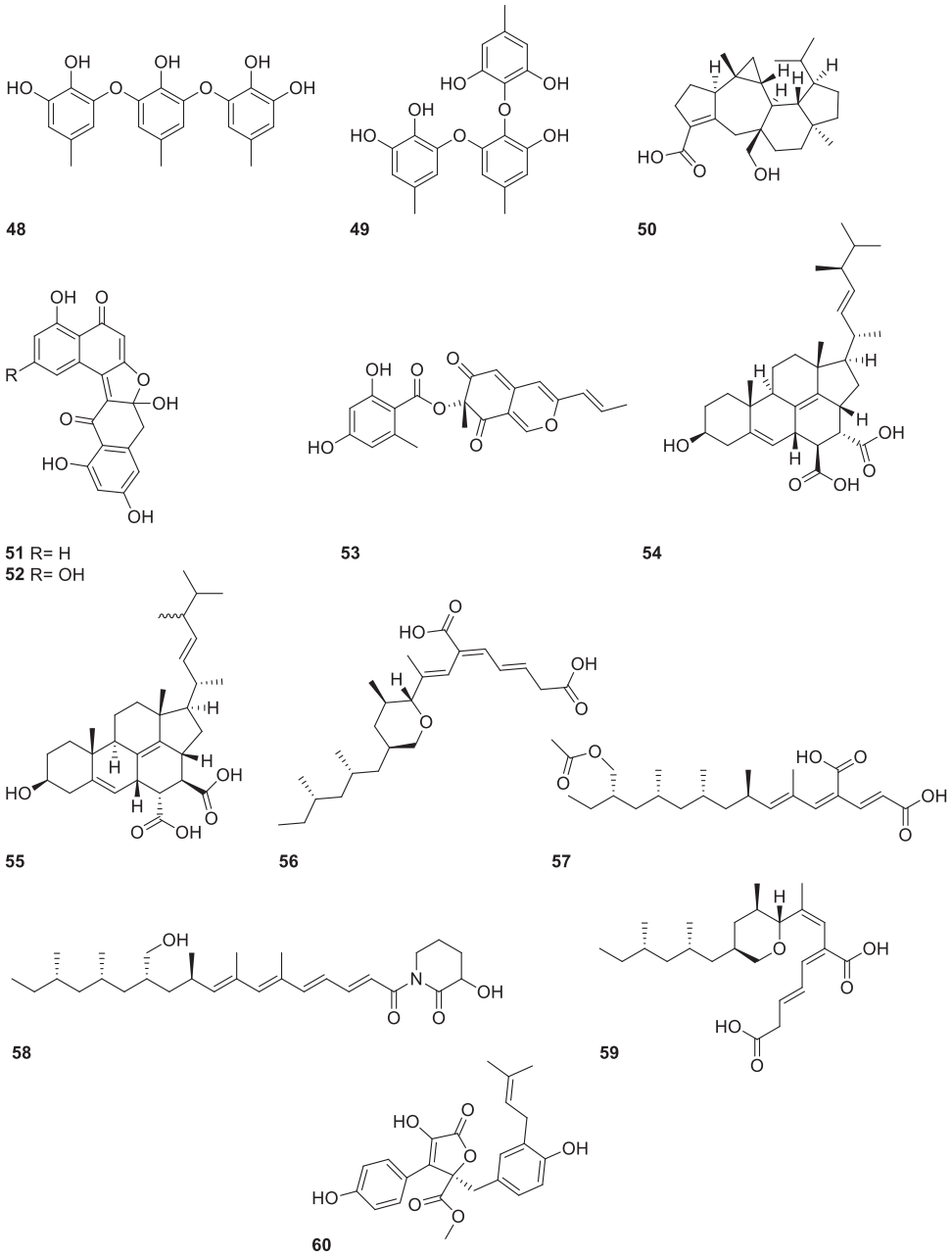

Fig. 11. Chemical structure of inhibitors targeting enzymes involved in immune escape mechanisms, extracted from *Aspergillus* spp.

activity against MptpB, with an IC_{50} value of 2.2 μM [93]. During an investigation on the chemical constituents of the same fungus, Xiao et al. extracted new and known compounds and tested them for their potential ability to inhibit MptpB. (±)-Asperlones A (**51**) and B (**52**) and (−)-mitorubrin (**53**) showed comparable activities to **50**, with IC_{50} values of about 4 μM (**51**: $IC_{50} = 4.2$ μM; **52**: $IC_{50} = 4.3$ μM; **53**: $IC_{50} = 4.0$ μM) [94]. Liu and co-workers isolated two Diels-Alder additive steroids, ergosterdiacids A (**54**) and B (**55**), from *Aspergillus* sp. DM29; these NPs were found to be active against MptpB, but with lower IC_{50} values (15.1 and 30.1 μM, respectively) with respect to **50** [95]. The same research group also evaluated the activity of few polypropionate derivatives from *Aspergillus fischeri*, finding that compounds **56**–**59** exhibited higher inhibitory activities with respect to the steroids derivatives, with IC_{50} values in the range of 4.0–11.0 μM. Kinetic experiments classified all these compounds as noncompetitive inhibitors of MptpB [96], similarly to the butyrolactone **60** isolated by Luo et al. from *Aspergillus terreus* SCSIO 41008, which showed an IC_{50} value of 5.11 μM [97].

Xia and co-workers reported the MptpB inhibition effects of several anthraquinone derivatives, extracted from the mangrove fungus *Alternaria* sp. SK11 [98]. Among them, the best activity was exhibited by (+)-aS-alterporriol C (**61**), which showed an IC_{50} value of 8.70 μM. Investigations on the fungus *Diaporthe* sp. SYSU-HQ3, isolated by Cui et al. from the mangrove plant *Excoecaria agallocha*, led to the identification of diaporisoindole (**62**) and tenellone C (**63**), active against MptpB with IC_{50} values of 4.2 μM and 5.2 μM, respectively [99]. Interestingly, the stereochemistry of **62** was found to be crucial for the activity: the **(*S*)-62** enantiomer displayed an IC_{50} of 4.2 μM, while **(*R*)-62** was still not active at a concentration of 50 μM. The kinetic analysis demonstrated that **(*S*)-62** was an uncompetitive inhibitor, while tenellone C (**63**) acted as a competitive inhibitor of this enzyme. Remarkably, both **62** and **63**

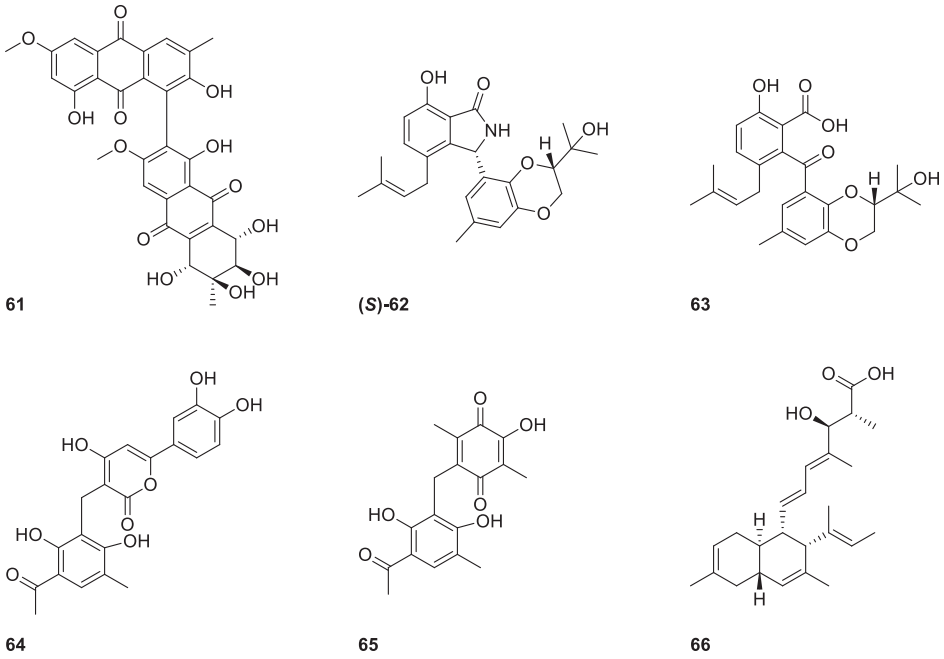

Fig. 12. Chemical structure of inhibitors targeting enzymes involved in immune escape mechanisms, extracted from marine fungi.

showed no activity against the human congener PPTP1B, proving to be selective for the mycobacterial PTP [99]. Further investigations by Li et al. on the mangrove-derived fungus *Penicillium dipodomyicola* led to the identification of peniphenones **64** and **65**, exhibiting a strong inhibitory effect against MptpB, with IC_{50} values of 0.16 μM and 1.37 μM, respectively [100]. Due to their promising activities and the uniqueness of their structures, these compounds could represent a new class of leads for the development of anti-TB drugs. In a recent study, a chemical investigation on the anemone-derived fungus *Fusarium graminearum* led to the identification of fusarielin M (**66**), a selective competitive inhibitor of MptpB (K_i = 1.03 μM), displaying a good *in vitro* anti-TB activity (MIC value = 12.3 mg/L) [101].

From the plant world, the most potent natural competitive inhibitors of MptpB were extracted from *Morus nigra.* Previously, Mascarello et al. isolated the Diels-Alder-type adduct Kuwanol E (**67**) (IC_{50} = 1.9 μM, K_i = 1.6 μM) [102]. More recently, two competitive inhibitors, Kuwanon G (**68**) and Kuwanon H (**69**), were discovered. These compounds exhibited IC_{50} values of 0.83 and 0.36 μM and K_i values of 0.39 μM and 0.20 μM, respectively. Both showed MICs of 32 μg/mL against *Mtb* H37Ra, together with a moderate specificity for MptpB over MptpA and PTP1B, and a good selectivity over lymphoid-tyrosine phosphatase (Lyp) and PTP-PEST [103]. The same research group also screened an in-house library of NPs, disclosing the non-competitive inhibitors **70**–**72**, slightly active against MptpB, but quite selective for this enzyme (**70**: IC_{50} = 26.7 μM; **71**: IC_{50} = 5.4 μM; **72**: IC_{50} = 13.4 μM) [102].

Two potent plant-derived inhibitors of MptpB were also reported in a patent by Chen and co-workers: flavonoid glycosides **73** and **74**, isolated from sweet potato, showed IC_{50} values of 4.2 and 6.0 μM, respectively [104].

Overall, the analysis of recent literature data revealed a strong prevalence of studies focused on MptpB rather than MptpA. The main reason for this imbalance is likely related to the significant sequence identity of MptpA to human PTPs, making it a far less attractive enzyme compared to its congener MptpB, which exhibits only a 6% similarity to the human PTP1B [105]. Regardless, the selectivity is a major hurdle for the development of all PTP inhibitors: hence, the discovery of novel candidates against these mycobacterial enzymes should always be supported by extensive data, proving their specificity *vs* a large panel of human analogues.

PknG is a virulence factor in *Mtb*, required for the inhibition of the phagolysosomal fusion. In this framework, Chen and co-workers tested sclerotiorin (**75**), an NP isolated primarily from *Penicillium sclerotiorum*, against this enzyme, finding IC_{50} and K_i values of 76.5 μM and 27.2 μM, respectively [106]. Despite the modest potency of this inhibitor, further biological tests evidenced that **75** blocked PknG autophosphorylation and reduced the growth of intracellular mycobacteria in a dose-dependent manner without provoking significant side effects on mammalian cells. Moreover, the fact that **75** is effective only on intracellular microorganisms may prevent the development of resistant strains. This compound may also be extremely efficient *in vivo*, since it would seem to be able to inhibit the mycobacterial kinases in the cytoplasm of the macrophage, without having to cross the *Mtb* cell wall. Notably, this considerable advantage is also shared by MptpA and MptpB inhibitors, which can act on the PTPs outside of the pathogen. The association of **75** with RIF resulted in a reduction of the mycobacterial growth, directly proportional to the concentration of **75**. Therefore, the moderate PknG inhibitory activity, the ability to reduce mycobacterial growth inside macrophages, and the low cytotoxicity of **75** strongly support the use of this compound in antitubercular therapy [106,107].

2.4. NPs interfering with metabolic pathways (D)

The treatment of TB is made extremely difficult by the presence of metabolically quiescent bacteria within host lesions. Frequently, these bacilli are not susceptible to traditional anti-TB therapeutics. Therefore, the identification of NPs able to block enzymes involved in the unique metabolism of *Mtb* is crucial to develop new drugs against persistent and latent TB infections. Promising targets were identified in many metabolic pathways. The antitubercular NPs here reported inhibit the following druggable enzymes: carbonic anhydrases (CAs), 1-deoxy-D-xylulose 5-phosphate reductoisomerase (Dxr), and CoA transferase (CoAt). The chemical

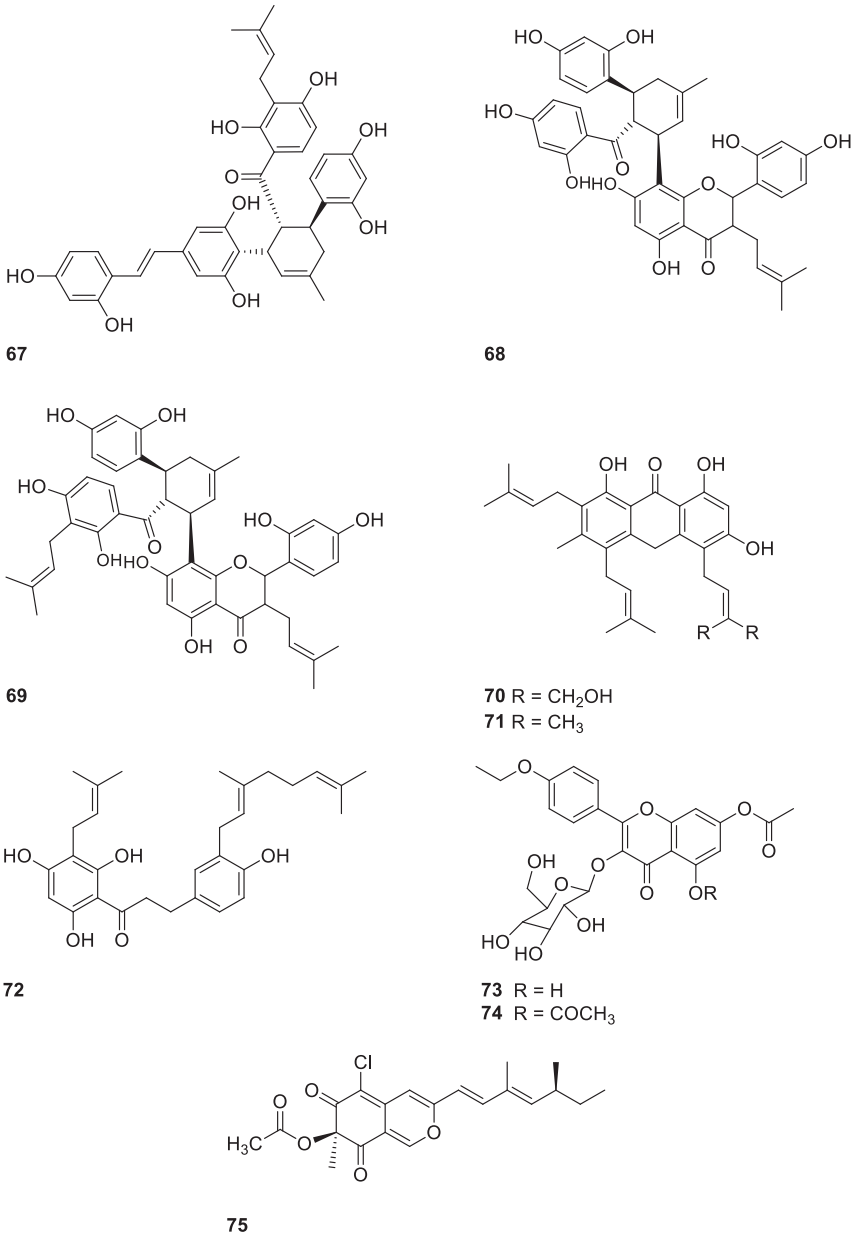

Fig. 13. Chemical structure of inhibitors targeting enzymes involved in immune escape mechanisms, extracted from plants.

structures of the selected NPs belonging to this class are shown in Fig. 14.

CAs catalyze the reversible hydratation of carbon dioxide to generate both bicarbonate and hydrogen ions for the regulation of pH homeostasis, which is essential for the survival of the bacterium. In particular, the soluble mycobacterial CAs Rv3588c and Rv1284 belong to a different class of CAs to those found in humans, making them attractive drug targets [108]. In 2017, Dallaston et al. selected the NP 2-methoxy-naphthoquinone (**76**) by screening the Davis Open Access Compound Library. Compound **76**, isolated from the plant *Impatiens balsamina* L., showed a specific non-classical inhibitory activity against CA Rv1284 (IC_{50} = 2.3 μM) and no significant effects on the human CA-II (relative activity about 104%) [109]. This inhibitor probably exploits the smaller volume of the catalytic sites of Rv1284 and Rv3588c (7 A^3 in Rv1284) compared to those of human α-CAs (100 A^3) to induce a selective inhibitory activity [110]. Further studies focusing on the SAR of non-carbonyl derivatives of **76** are still ongoing [109]. Few years before, von Gnielinski N. et al. screened an in-house library and identified three inhibitors of CA Rv3588c: ianthelliformisamine C (**77**), from the sponge *Suberea ianthelliformis*, spermatinamine (**78**), from the sponge *Pseudoceratina* sp., and (+)-mispyric acid (**79**), from the stem bark of the rainforest plant *Mischocarpus*. Compounds **77**–**79** displayed inhibitory effects against CA Rv3588c with K_i values of 16 μM, 23 μM, and 10 μM, respectively. In particular, **77** and **78** showed good MIC_{90} values (12.5 and 6.3 μM, respectively), while **79** was less active against *Mtb* (MIC_{90} > 50 μM), probably due to its high lipophilic nature, which may reduce its cell permeability [111]. Previously, Davis and co-workers screened a library of phenolic NPs for their inhibitory activity against the CAs encoded by genes Rv3273 and Rv1284 [112]. Among the tested derivatives, compound **80** exhibited the best inhibitory activity against both Rv3273 and Rv1284, with K_i values of 0.89 and 0.80 μM, respectively. However, it suffered from a low selectivity, being also active against the human CA-II (K_i = 12.1 μM). For this reason, **80** was not developed further; conversely, (−)-dihydroguaiaretic acid (**81**) found in the

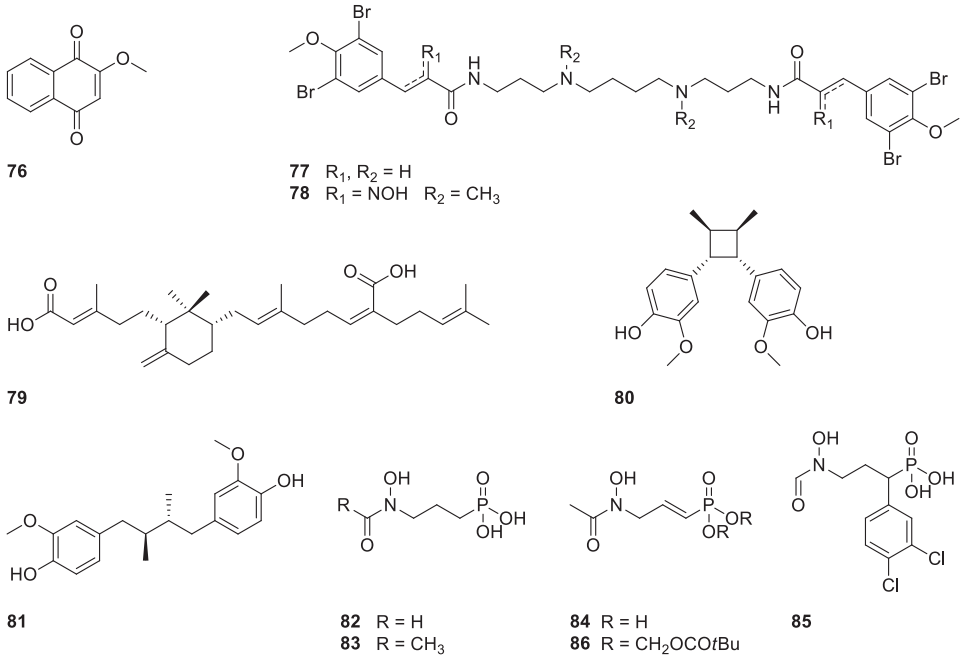

Fig. 14. Chemical structure of inhibitors targeting enzymes involved in metabolic pathways.

creosote bush of *Larrea tridentata*, was selected as the new lead on the basis of the results of the activity and selectivity assays. This compound emerged as a potent CA inhibitor (CA Rv3273: K_i = 9.10 μM; CA Rv1284: K_i = 0.85 μM) and showed good selectivity, having K_i values of 131 μM and 307 μM, respectively, against human CA II-I [112]. In addition, **81** showed growth inhibitory activities against MDR (MIC in the range: 12.5—50 μg/mL) and H37Rv (MIC of 50 μg/mL) strains [113]. All the reported molecules represent innovative chemical entities that explore different chemical scaffolds compared to the classical CA inhibitors, in which the sulfonamide moiety provides an anchorage to the zinc ion present in the active site. While **76** can exploit the catalytic sites of the CAs due to its small volume, **77**—**79**, and **81** have an extended shape that restricts their access to the active site of β-CA. Their inhibitory effect is most likely due to monomerization or to the induction of conformational changes in the outer region of the active site [111,112]. Later, Clemente-Soto et al. performed gene expression tests from total RNA obtained from *Mtb* H37Rv treated with **81** using microarray technology, validated by quantitative real-time polymerase chain reaction (PCR) [113]. The analyses evidenced the overexpression of the Rv3551 gene, which encodes for the α subunit of the mycobacterial CoAt. These results were supported by molecular docking analyses that showed stable interaction between **81** and the active site of CoAt. The inhibition of this enzyme resulted in the accumulation of geraniol and 1- and 2-methylnaphtalene inside bacteria, causing membrane destabilization and the consequent death of the pathogen. This mycobacterial pathway is particularly attractive because it is absent in the human host. Moreover, this mechanism could represent an innovative approach, in which essential homeostasis conditions are simultaneously targeted to improve the microbicidal effects of the drug.

Fosmidomycin (**82**), originally isolated from culture broths of bacteria of the genus *Streptomyces*, and its close derivative FR900098 (**83**) were found to interfere with the first and second committed steps of the nonmevalonate pathway (NMP) of isoprene biosynthesis, catalyzed by Dxr (IC_{50} of 0.44 μM and 2.39 μM, respectively). Considering that many pathogenic organisms rely exclusively on NMP for the biosynthesis of isoprenoids while humans do not, the enzymes involved in this route have been recently explored as new therapeutic targets. Jackson and co-workers found out that **82** and **83** can mimic the polar character of the natural substrate of Dxr [114]. Since these NPs lacked antibacterial activity (MIC > 500 μg/mL), probably because of their poor uptake, lipophilic analogues were synthetized to obtain activity against *Mtb*. These derivatives retained the key structural features of the parent compounds, namely a phosphonate, a retro-hydroxamic acid, and an *n*-propyl carbon chain linking the nitrogen and phosphorus atoms. The α/β-unsaturated analog **84** emerged as the most potent inhibitor of Dxr, with an IC_{50} of 1.07 μM, but failed in whole-cell assays (MIC > 200 μg/mL) [114]. Furthermore, among the α-aryl substituted analogues of **82** synthesized by Andaloussi et al., the best inhibitor (**85**) showed an IC_{50} of 0.15 μM against Dxr, but still lacked activity in mycobacterial growth assays [115]. The most interesting compound (**86**) was actually the more lipophilic pivaloyl ester of **84**, which was also effective against *Mtb* (MIC of 9.4 μg/mL), while retaining a comparable activity against the enzyme [114].

3. Conclusions and outlook

The great mass of research works devoted to the discovery of antitubercular NPs selectively targeting mycobacterial enzymes is here reported. A total of 86 NPs deriving from plants, fungi, bacteria, and marine species have been found to possess anti-mycobacterial properties, also against resistant isolates. Most of the compounds interfere with enzymes involved in the biosynthesis of the cell wall and in protein metabolism. Considering the wide range of enzymes mediating survival and virulence processes in *Mtb*, the selectivity of most of the reported inhibitors is one of the great benefits of these compounds. In addition, the study of NPs has greatly contributed to expand the knowledge about the role of the selected targets in TB infection, as well as to identify and develop new semisynthetic inhibitors.

The majority of NPs targeting enzymes involved in the synthesis and metabolism of DNA, RNA, and proteins derive from plants. Among them, the catechin **2** is the most potent compound, selectively inhibiting DHFR (MIC ≈ 4 μM). Among bacteria-derived NPs, the myxopyronin **8** emerged as an excellent RNAP inhibitor

(IC_{50} = 100 nM) endowed with good antibacterial properties (MIC ≈ 3.7 μM). Importantly, when co-administered with RIF, it exhibited a synergistic antibacterial activity. Several studies investigated Clp complex as an innovative anti-TB target: among the compounds acting on this enzyme, ecumicin (**16**) exhibited an outstanding and selective inhibitory activity (MIC = 160 nM), which was retained against resistant strains. Notably, the compound proved to be able to completely block *Mtb* growth in mice models.

The plant world is again a key source of NPs capable of interfering with the cell wall and fatty acid biosynthesis. Several coumestan derivatives recently emerged as promising enzymatic inhibitors, active against the validated drug target Pks13. In particular, **46** showed excellent activity against both drug-susceptible and drug-resistant *Mtb* strains (MIC ≈ 0.004 μg/mL), a favorable human microsomal stability, selectivity against human cells, as well as oral bioavailability in mice, displaying an 8-fold higher activity than INH *in vivo*. Among the semisynthetic derivatives, several dihydrosansanmycins, structurally related to antibiotic compounds produced by the soil bacterium *Streptomyces* sp. SS, exhibited excellent inhibitory activities against *Mtb*. The best compound was **33**, a very potent MraY inhibitor (IC_{50} = 41 nM) endowed with a MIC_{50} value of 0.04 μM. Compounds **36** and **37**, inhibiting both UGM and TBNAT, represent innovative scaffolds for the development of a dual-target approach. However, further studies are necessary to better investigate the biological activity of these promising compounds.

Concerning the inhibitors of enzymes involved in immune escape mechanisms, the plant derivative **69** and the fungus-derived **66** are the most promising, due to their effective inhibition of MptpB. Despite their modest *in-vitro* potency, they reduced the growth of intracellular mycobacteria without provoking significant side effects on mammalian cells. Furthermore, due to the extracellular localization of MptpB, the enzymatic inhibitors do not have to cross the bacterial cell wall, making them innovative and promising lead compounds.

Finally, among the reported inhibitors of enzymes involved in metabolic pathways, it is important to evidence that the plant derivative **81** inhibited both Rv3273 and Rv1284 CAs, displaying high selectivity for *Mtb* and a promising activity against MDR strains (MIC range: 12.5–50 μg/mL).

Starting from the invaluable research works reported herein, the activity, selectivity, and safety profile of NPs could be potentially optimized through the development of semisynthetic and synthetic derivatives, carefully designed to enhance the efficacy, and lower the toxicity of the newly developed anti-TB drug candidates. Advances in pre-clinical microbiological studies are expected in the next future to pave the way for the use of NPs as novel scaffolds to revolutionize the therapy of TB.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

All reported compounds are supplied as .mol files.

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